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Blood Plasma Sample Preparation Method for the Assessment of Thyroid Hormone-Disrupting Potency in Effect-Directed Analysis

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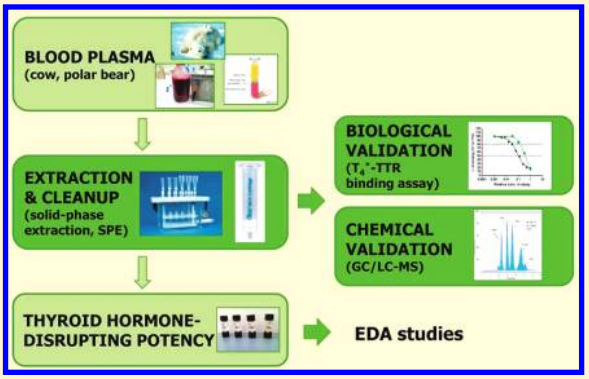
Supporting Information

ABSTRACT: A sample preparation method combining solid-phase extraction (SPE) and liquid−liquid extraction (LLE) was developed to be used in Effect-Directed Analysis (EDA) of blood plasma. Until now such a method was not available. It can be used for extraction of a broad range of thyroid hormone (TH)-disruptors from plasma with high recoveries. Validation of the method using spiked cow plasma showed good recoveries for hydroxylated polybrominated diphenyl ethers (OH-PBDEs; 93.8 ± 19.5%), hydroxylated polychlorinated biphenyls (OH-PCBs; 93.8 ± 15.5%), other halogenated phenols (OHPs; 107 ± 8.1%), and for short-chain (<8 C-atoms) perfluoroalkyl substances (PFASs; 85.2 ± 24.6%). In the same extracts, the potency of the compound classes spiked to the cow plasma to competitively bind to transthyretin (TTR) was recovered by 84.9 ± 8.8%. Furthermore, the SPE-LLE method efficiently removed endogenous THs from the extracts, thereby eliminating their possible contribution to the binding assay response. The SPE-LLE method was applied to polar bear plasma samples to investigate its applicability in future EDA studies focusing on TH-disrupting compounds in this top predator species that is exposed to relatively high levels of bioaccumulating pollutants. A first screening revealed TTR-binding potency in the polar bear plasma extracts, which could be explained for 60−85% by the presence of OH-PCBs.



INTRODUCTION

Persistent organic pollutants (POPs) in the environment can bioaccumulate and biomagnify in the foodchain.[1] POPs are consequently found in significant concentrations in adipose tissue and blood of top predators.[2] The number of reports on levels of blood accumulative pollutants (BAP) in humans[3] and wildlife[1,2] is rapidly increasing. These chemicals, such as polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), hydroxylated PBDEs (OH-PBDEs), hydroxylated PCBs (OH-PCBs) and other halogenated phenols (OHPs), are known to interfere with the thyroid hormone (TH) system.[4] TH-disrupting compounds may compete with thyroxine (T_4) binding to transthyretin (TTR), one of the T_4-transport proteins.[4,5] Other compounds such as perfluoroalkyl substances (PFASs) have no structural similarity with THs but have a TTR-binding potency in the order of one-tenth of the endogenous T_4.[6] TTR-binding compounds can reduce the concentrations of THs in the blood circulation of experimental animals[7] as well as in humans[8] and wildlife,[9] but the lowest dose of these contaminants causing this effect is not well established.[4] Decreased TH levels in blood might lead to subclinical hypothyroidism in adults. Furthermore, a normal level of THs is crucial for development of the central nervous system during gestation and for general development in childhood.[4] In wildlife there is concern that TH-disruption may have a negative effect on fitness of top predator species, such as the polar bear (*Ursus maritimus*).[10]

To identify known and unknown TH-disruptors in complex environmental matrices (e.g., blood plasma), Effect-Directed Analysis (EDA) - the combined and iterative use of biological and analytical chemical techniques to direct chemical analysis to those compounds that actually have biological activity[11] - is a promising approach. For successful EDA studies of blood plasma a gentle, nondestructive, nondiscriminating sample treatment is one of the key factors to broaden the scope of EDA from abiotic to biotic matrices.

The objective of the present study was to develop and validate a method combining solid-phase extraction (SPE) and liquid−liquid

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Table 1. Overview of the Six Spiking Mixtures Used to Spike Cow Plasma Samples[k]

spiking mixture	compound	chemical name	CAS number	MW (g/mol)	spiking level in plasma (μM)	molar T_4-REP factors	reference REP-factors
1. OH-PCBs	4-OH-CB 107[a]	4-hydroxy-2,3,3′,4′,5-pentachlorobiphenyl	n.a.	342.44	3.4×10^{-2}	3.5	this study
	4-OH-CB 118[b]	4-hydroxy-2,3′,4,4′,5-pentachlorobiphenyl	n.a.	342.44	3.4×10^{-2}	4.1	this study
	4′-OH-CB 130[b]	4′-hydroxy-2,2′,3,3′,4,5′-hexachlorobiphenyl	n.a.	376.88	3.1×10^{-2}	3.7	this study
	4-OH-CB 187[a]	4-hydroxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl	n.a.	411.33	2.8×10^{-2}	4.0	this study
2. OH-PBDEs	2′-OH-BDE-66[b]	2′-hydroxy-2,3′,4,4′-tetrabromodiphenylether	n.a.	501.8	4.5×10^{-2}	6.5×10^{-1}	Hamers et al., 2008[12]
	3-OH-BDE-47[b]	3-hydroxy-2,2′,4,4′-tetrabromodiphenylether	n.a.	501.8	4.6×10^{-2}	4.0	Hamers et al., 2008[12]
	4′OH-BDE-49[b]	4′-hydroxy-2,2′,4,5′-tetrabromodiphenylether	n.a.	501.8	3.3×10^{-2}	3.5	Hamers et al., 2008[12]
	4-OH-BDE-47[b]	4-hydroxy-2,2′,3,4′-tetrabromodiphenylether	n.a.	501.8	4.6×10^{-2}	3.9×10^{-1}	Hamers et al., 2008[12]
	5-OH-BDE-47[b]	5-hydroxy-2,2′,4,4′-tetrabromodiphenylether	n.a.	501.8	3.0×10^{-2}	3.0	Hamers et al., 2008[12]
3. PCBs and PBDEs	BDE 28[c]	2,4,4′-tribromodiphenyl ether	41318-75-6	485.8	3.1×10^{-2}	-	Hamers et al., 2006[13]
	BDE 47[c]	2,2′,4,4′-tetrabromodiphenyl ether	5436-43-1	485.8	3.4×10^{-2}	2.2×10^{-3}	Hamers et al., 2006[13]
	BDE 49[c]	2,2′,4,5′-tetrabromodiphenyl ether	446255-38-5	485.8	3.1×10^{-2}	2.2×10^{-3}	Hamers et al., 2006[13]
	BDE 100[c]	2,2′,4,4′,6-pentabromodiphenyl ether	32534-81-9	564.7	3.1×10^{-2}	-	Hamers et al., 2006[13]
	PCB 138[d]	2,2′,3,4,4′,5′-hexachlorobiphenyl	35065-28-2	360.9	1.3×10^{-1}	$<3.8 \times 10^{-3}$	Hamers et al., 2011[14]
	PCB 153[d]	2,2′,4,4′,5,5′-hexachlorobipenyl	35065-27-1	360.9	1.3×10^{-1}	$<3.8 \times 10^{-3}$	Hamers et al., 2011[14]
	PCB 170[d]	2,2′,3,3′,4,4′,5-heptachlorobiphenyl	35065-30-6	395.3	1.2×10^{-1}	$<3.8 \times 10^{-3}$	Hamers et al., 2011[14]
	PCB 180[d]	2,2′,3,4,4′,5,5′-heptachlorobiphenyl	39635-31-9	395.3	1.2×10^{-1}	$<3.8 \times 10^{-3}$	Hamers et al., 2011[14]
4. PFASs	PFBA[e]	perfluorobutyric acid	375-22-4	214.0	6.1×10^{-2}	n.d.	Weiss et al., 2009[6]
	PFPeA[f]	perfluoro-n-pentanoic acid	2706-90-3	264.0	5.1×10^{-2}	1.8×10^{-1}	this study
	PFHxA[f]	perfluorohexanoic acid	307-24-4	314.0	4.2×10^{-2}	7.0×10^{-3}	Weiss et al., 2009[6]
	PFHpA[e]	perfluoroheptanoic acid	375-85-9	364.1	3.5×10^{-2}	3.9×10^{-2}	Weiss et al., 2009[6]
	PFOA[f]	perfluorooctanoic acid	335-67-1	414.0	3.1×10^{-2}	6.4×10^{-2}	Weiss et al., 2009[6]
	PFNA[g]	perfluorononanoic acid	375-95-1	464.0	2.8×10^{-2}	2.2×10^{-2}	Weiss et al., 2009[6]
	PFDcA[e]	perfluorodecanoic acid	335-76-2	514.0	2.8×10^{-2}	7.0×10^{-3}	Weiss et al., 2009[6]
	PFUnA[g]	perfluoroundecanoic acid	2058-94-8	563.9	2.3×10^{-2}	3.0×10^{-3}	Weiss et al., 2009[6]
	PFDoA[g]	perfluorododecanoic acid	307-55-1	614.0	2.2×10^{-2}	1.0×10^{-3}	Weiss et al., 2009[6]
	PFTrA[e]	perfluorotridecanoic acid	72629-94-8	664.0	1.9×10^{-2}	6.4×10^{-2}	this study
	PFTdA[e]	perfluorotetradecanoic acid	376-06-7	713.9	1.8×10^{-2}	2.0×10^{-3}	Weiss et al., 2009[6]
	PFBS[g]	perfluorobutane sulfonate	2795-39-3	300.0	3.8×10^{-2}	3.0×10^{-3}	Weiss et al., 2009[6]
	PFHxS[h]	perfluorohexane sulfonate	3871-99-6	400.0	3.2×10^{-2}	8.5×10^{-2}	Weiss et al., 2009[6]
	6:2 FTS[i]	6:2 fluorotelomersulfonate	27619-97-2	427.0	2.5×10^{-2}	n.d.	this study
	PFOS[h]	perfluorooctane sulfonate	2795-39-3	500.0	2.5×10^{-2}	6.5×10^{-2}	Weiss et al., 2009[6]
	PFOSA[i]	perfluorooctanesulfonamide	754-91-6	499.2	2.7×10^{-2}	1.0×10^{-2}	Weiss et al., 2009[6]
5. OHPs	2.4.6-TCP[j]	2,4,6-trichlorophenol	88-06-2	197.5	4.7×10^{-1}	3.3×10^{-1}	Van den Berg et al., 1990[15]
	2.4.6-TBP[j]	2,4,6-tribromophenol	118-79-6	330.8	2.9×10^{-1}	3.0	this study
	PCP[g]	pentachlorophenol	87-86-5	266.3	3.4×10^{-1}	1.7	Van den Berg et al., 1990[15]
	triclosan[g]	5-chloro-2-(2,4-dichlorophenoxy)phenol	3380-34-5	289.5	3.5×10^{-1}	1.0×10^{-2}	Crofton et al., 2007[18]
6. Combined mixture	OH-PCBs/OH-PBDEs/OHPs (The final concentration of the individual compounds in the combined mixture was the same as in the mixtures 1, 2, and 5, respectively.)						

[a] Greyhound (Birkenhead, UK). [b] Synthesized at Stockholm University by Prof. Dr. Åke Bergman. [c] Chiron (Trondheim, Norway). [d] ULTRA Scientific (N. Kingstown, USA). [e] ABCR GmbH & Co (Karlsruhe, Germany). [f] Acros Organics (Geel, Belgium). [g] Sigma (Zwijndrecht, The Netherlands). [h] Fluka (Zwijndrecht, The Netherlands). [i] Wellington (Guelph, Canada). [j] Riedel de Haën (Seelze, Germany). [k] The combined mixture did not contain PCBs/PBDEs (mixture 3) and PFASs (mixture 4). The spiking level in plasma indicates the concentration of the individual compounds.

extraction (LLE) for extraction of a broad range of known and unknown TH-disrupting compounds from plasma samples. Recoveries of a wide selection of TH-disrupting compounds from spiked cow plasma were chemically determined by gas and liquid chromatography (GC and LC) coupled to mass spectrometry (MS) and recoveries of their competitive TTR-binding activities were biologically determined in the T_4*-TTR binding assay.[5] Furthermore, the total T_4 (TT_4) concentration in cow plasma before and after the extraction was measured to estimate the T_4 removal capability of the SPE-LLE method. Extracts of cow plasma samples spiked with OH-PCBs and OH-PBDEs were also fractionated using normal-phase high performance liquid chromatography (NP-HPLC) to separate xenobiotic TH-disrupting compounds from endogenous THs that could affect the bioassay results. Finally, two polar bear plasma extracts were prepared using the validated SPE-LLE method and tested in the T_4*-TTR binding assay to demonstrate the applicability of the developed sample preparation method in further EDA studies.

MATERIALS AND METHODS

Plasma Samples and Spiking Mixtures. Cow plasma was used for method validation because no or only very low levels of

contaminants were expected in this type of plasma compared to plasma from top predators, such as polar bears. Polar bear blood samples were collected from two adult (8 and 9 year old) female bears at Svalbard (Norway) in April 2008. Two mL of each plasma sample was extracted and tested in the radioligand T_4*-TTR binding assay. These samples were selected based on their moderate TT_4 level (8 and 20 nM, respectively), high plasma lipid levels (1.5% for both), and knowledge of the plasma OH-PCB levels as determined within a much larger study (BearHealth, Web site: http://biologi.no/bearhealth-eng.htm). Further information on cow plasma preparation and on the sampling, extraction, and chemical analysis of the polar bear samples can be found in the Supporting Information.

Spiking mixtures were prepared of well-known classes of TH-disrupting compounds, such as OH-PCBs, OH-PBDEs, and OHPs in hexane (J.T. Baker, The Netherlands) and PFASs in methanol (MeOH; J.T. Baker). In addition, spiking mixtures of the parent PCB and PBDE compounds were prepared in hexane. Three mL of cow plasma was spiked in a final concentration range of 0.018−0.47 μM with 30 μL of the different spiking mixtures. The final spiking concentration range was determined based on the environmentally relevant concentrations and on the analytical detection limits of the spiking compounds. In order to better mimic environmental circumstances (e.g., that BAPs might bind to blood plasma proteins) a low spiking volume (30 μL) was used to avoid direct protein precipitation by the organic solvent of the spiking mixtures. The PFAS mixture and the PFAS-spiked plasma samples were stored in polypropylene tubes to avoid interaction with glass surfaces. An overview of the individual test compounds, the mixtures and the concentrations of the individual compounds in the blood plasma after spiking is given in Table 1. Spiked cow plasma samples, cow plasma blanks (3 mL), and procedure blanks (3 mL HPLC-grade water) were prepared in triplicate, whirl-mixed, and placed in the refrigerator (at 4 °C) for overnight equilibration to enable the formation of possible bindings between the TH-disruptors and plasma proteins. To test the influence of the sample matrix on the recoveries, an additional spiking experiment was performed with 3 mL of HPLC-grade water that was spiked with 30 μL of the OH-PBDE mixture.

Sample Preparation. An overview of the experimental design of the validation study is shown in Figure 1. The sample preparation method consists of four steps: protein denaturation, SPE, LLE, and NP-HPLC fractionation.

Protein Denaturation. To denaturate plasma proteins, formic acid (HCOOH, 99% v/v; Sigma) in 2-propanol (Sigma) (4:1, v/v) was added in a 1:1 (v/v) ratio to 3 mL of procedure blanks, spiked and blank cow plasmas, and to 2 mL of polar bear plasmas following the method described by Thomsen et al.[3] with minor modifications. The samples were whirl-mixed, sonicated in an ultrasonic bath for 10 min, and stored in the dark for 50 min at room temperature. Subsequently, 3 mL of water/2-propanol (4:1, v/v) was added to each sample (2 mL to the polar bear plasma), and the resulting mixture was sonicated for another 10 min. Finally, to reduce the influence of the organic solvent on the SPE procedure, the samples were diluted with water until the organic solvent percentage (isopropanol and solvent of the spiking mixture) was less than 5%. Typically, the total volume before SPE was 25 mL. The obtained plasma samples were clear and required no centrifugation step for pellet separation before extraction.

Solid-Phase Extraction (SPE) and Clean Up. The Oasis MCX cartridge (150 mg, 6 mL) was selected for this study, because highest chemically determined recoveries of TH-disrupting compounds was obtained for this cartridge in a pilot experiment comparing seven different cartridges (Supporting Information). Oasis MCX cartridges were conditioned with MeOH (3 mL) and equilibrated with water (3 mL) before the treated plasma samples were loaded. Solvents and samples were passed through the cartridges dropwise. The cartridges were washed with water containing 1.8% HCOOH (3 mL). After the washing step the SPE sorbent material was dried completely before elution. The adsorbed compounds were eluted from the MCX cartridges with 4 × 0.75 mL MeOH (elution 1, E_1) followed by 4 × 0.75 mL MeOH containing 5% ammonium hydroxide (NH_4OH; Fluka, The Netherlands) (elution 2, E_2). Vacuum was only used after the washing step, before elution. The SPE procedure is summarized in Figure 1.

Liquid−Liquid Extraction (LLE). Using LLE, all E_1 and E_2 fractions were transferred into hexane that was more suitable for further analysis, except for the extracts of the plasma spiked with PFASs. Of the E_1 and E_2 fractions of these extracts, 50% was kept in MeOH, since the PFASs were analyzed on a triple quadrupole liquid chromatography electrospray ionization tandem mass spectrometer (LC-ESI-MS/MS, see Chemical Validation) using a MeOH:water (1:1, v/v) mobile phase. For LLE, the E_1 and E_2 fractions were transferred to 20 mL glass tubes with screw caps, and 5−7 mL of 4% phosphoric acid (H_3PO_4; Fluka) and 2 mL of hexane were added. The addition of H_3PO_4 improved the extraction of the hydroxylated compounds (data not shown). Subsequently, the samples were vortexed (1 min) and centrifuged (3 min; 1500 rpm). The upper hexane layers containing the compounds of interest were carefully separated and collected. The hexane fractions were then loaded onto a Pasteur pipet filled with dried sodium sulfate (Na_2SO_4, Promochem, Germany) to remove water, if present. The whole procedure, starting with the addition of 2 mL of hexane, was repeated another two times. For each fraction, the hexane layers were combined, and the extracts $E_{1,\,hex}$ and $E_{2,\,hex}$ were evaporated under nitrogen until a volume of 0.5 mL.

Normal-Phase High Performance Liquid Chromatography (NP-HPLC). To avoid interference of endogenous THs with the bioassay measurements, they were separated from the plasma extracts via NP-HPLC fractionation. Half of both the $E_{1,\,hex}$ and $E_{2,\,hex}$ fractions of the plasma spiked with OH-PCBs and OH-PBDEs was loaded on a μPorasil NP-HPLC column (7.8 mm ID, 300 mm) packed with 10-μm μPorasil (Waters Assoc., USA), using a mobile phase gradient of hexane, dichloromethane (DCM; Promochem), and acetonitrile (ACN; J.T. Baker) at a flow-rate of 5 mL/min, as described before.[17] The 0−40 min (F_1) and 41−49 min (F_2) NP-HPLC fractions were collected, resulting in the fractions E_1F_1, E_1F_2, E_2F_1, and E_2F_2 (Figure 1). In these fractions recoveries (chemical analysis) and competitive TTR-binding activities (T_4*-TTR assay) were determined. The analytes were expected to elute from the column in the first HPLC-fractions, the endogenous hormones in the second.[17] The other half of the extracts was kept unfractionated to compare the chemically determined recoveries and the biological activities of the fractionated and nonfractionated extracts. Fractionated and nonfractionated extracts were equally divided into two parts: half was exchanged into 50 μL of DMSO (Acros, Belgium) for testing in the T_4*-TTR binding assay and half was kept in 50 μL of hexane for chemical analysis.

Endogenous Total T_4 Levels. To check the capacity of the SPE procedure to remove endogenous T_4, TT_4 concentration was measured in the original cow plasma and in its E_1 fraction at the Academic Medical Center (AMC) Amsterdam using a radioimmunoassay, according to their validated protocol. The E_1

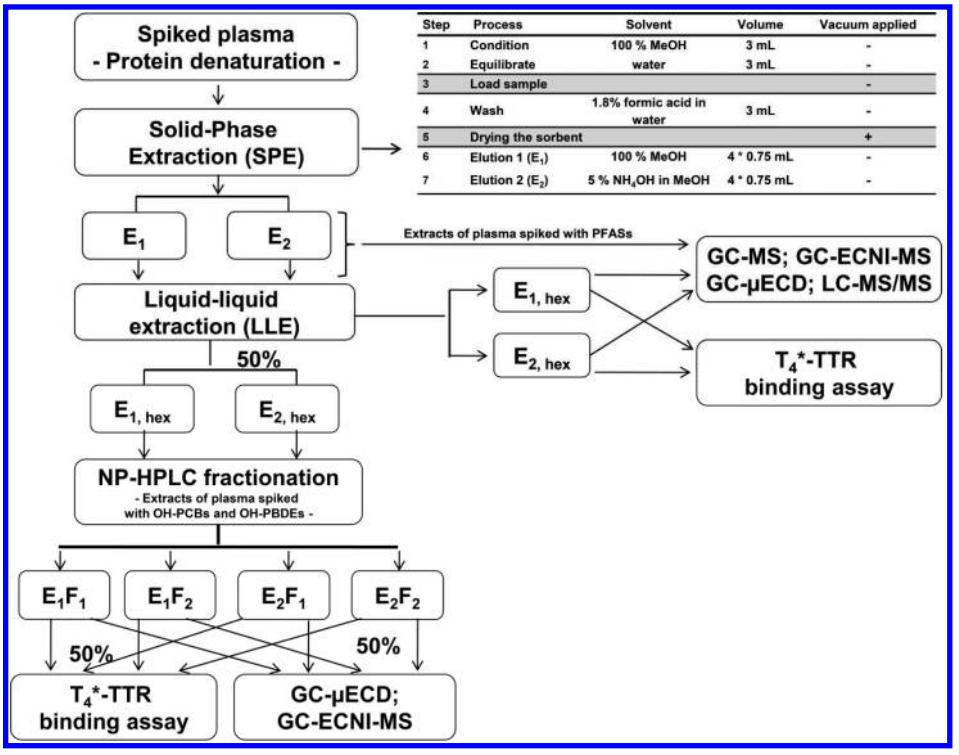

Figure 1. Complete experimental scheme of the SPE-LLE-HPLC extraction and cleanup method evaluation on Oasis MCX (150 mg/6 mL) cartridges.

fraction in MeOH was evaporated under nitrogen to almost dryness and dissolved in bovine serum (Gibco, The Netherlands) stripped with T500dextran and activated charcoal. As a control, TT_4 levels were also determined in stripped serum only. The initial TT_4 concentration in polar bear plasma was measured previously within the BearHealth project using a radioimmunoassay according to Braathen et al.[9]

Chemical Validation. For chemical analysis, internal standards are generally used to evaluate the analytical method performance, as for instance done by Thomsen et al.[3] However, to determine recoveries in biota extracts in bioassays or in an EDA setup, internal standards cannot be used due to their possible influence on the bioassay results. Thus, target compounds were quantified in all cases by external standard calibration by the same mixtures used for spiking.

Chemically determined recoveries of OH-PBDEs in the extracts of the spiked plasma were measured by gas chromatography−mass spectrometry (GC-MS) operated with electron capture negative ionization (ECNI) in the selected ion mode (SIM). Recoveries of OHPs were determined by GC-electron impact (EI)-MS operated in both SIM and full scan mode (scanning mass range: 50−400 m/z). The analysis of PCBs, PBDEs, and OH-PCBs was performed by GC with micro electron capture detection (μECD). PFASs were analyzed by LC-ESI-MS/MS. For more detailed method descriptions see the Supporting Information.

Bioassay Validation. To assess the competition between the TH-disrupting compounds in the extracts and the reference T_4 (Sigma) for binding to the T_4-transporting protein TTR (Sigma), the extracts were tested in the radioligand T_4*-TTR binding assay according to Lans et al.[5] with modifications as described by Hamers et al.[13] and Weiss et al.[6] The spiking mixtures were also tested directly in the assay to determine their activities in the absence of any sample matrix. The spiking mixtures, all fractions of the procedure blanks and of the extracts of the spiked and nonspiked plasma were tested in the bioassay in a dilution series (1−3−10−30−100). The extract of the plasma spiked with the combined mixture was tested in more dilutions (1−3−10−30−60−100−300) due to its expected higher activity. Dose−response curves of the reference compound T_4 were fitted using a sigmoidal fit with variable slope in GraphPad Prism (version 5.01 for Windows, GraphPad Software). TTR-binding activities of the extracts were expressed as T_4 equivalent (T_4-Eq) concentrations. For interpolation into the T_4 dose−response curve (Figure 2A), the most diluted sample was used that caused a response in the 20−50% inhibition window.

To calculate the theoretically expected TTR-binding activity of the spiking compounds (in terms of T_4-Eq concentrations), the concentration of each spiked compound was multiplied by its relative potency factor (REP-factor). The expected total TTR-binding activity of the extract was calculated by summation of the TTR-binding activities calculated for the individual compounds in the mixture. Finally, recoveries were determined by comparing the actually measured activity of the spiked plasma extracts to i) the calculated total activity of the spiked compounds and ii) to the actually measured activities of the spiking mixtures (after transfer to DMSO). Spiking concentrations and REP factors of the test compounds are listed in Table 1. REP factors were collected from previously published studies or were determined in the T_4*-TTR binding assay in this study (Table 1 and 4, Figure 2B). REP-factors were determined by the 50% inhibition concentrations (IC_{50}) of the reference compound (T_4) and of the test compound: $\text{REP-factor}_{\text{test comp}} = IC50_{T4}/IC50_{\text{test comp}}$.

Similarly, the theoretically expected activity of the polar bear plasma extract in the T_4*-TTR binding assay was calculated based on the measured OH-PCB levels and the REP-factors listed in Table 4.

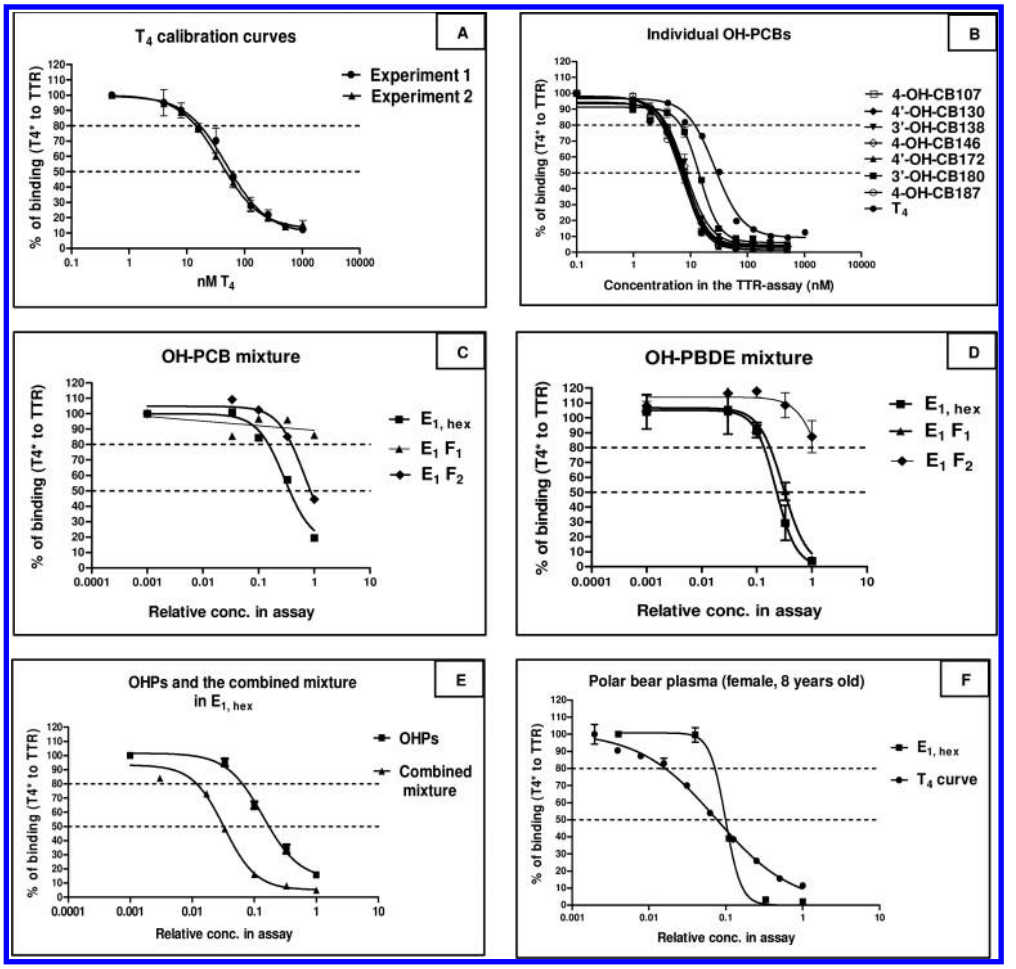

Figure 2. Competitive TTR-binding potency A) of the T_4 reference curve in different experiments, B) of the individual OH-PCB standards for REP-value determination, C) $E_{1,\,hex}$, E_1F_1 and E_1F_2 (sub)fractions of plasma spiked with OH-PCBs, D) $E_{1,\,hex}$, E_1F_1 and E_1F_2 (sub)fractions of plasma spiked with OH-PBDEs, E) fraction $E_{1,\,hex}$ of plasma spiked with OHPs and the combined mixture, F) fraction $E_{1,\,hex}$ of one of the polar bear plasma extracts. In A-B) the logarithmically scaled *X*-axes show the concentration of T_4 and OH-PCBs in the assay (nM) and in C−F) the logarithmically scaled *X*-axes show the relative concentration of T_4 and the analytes in the extracts based on the dilution of the extracts. The undiluted extracts correspond to 1. On the *Y*-axis of each figure the percentage (%) of binding of T_4* to TTR is indicated.

RESULTS AND DISCUSSION

Validation. *Recoveries Determined by Chemical Analysis.* For the plasma samples spiked with OH-PCBs and the OH-PBDEs, both the fractions $E_{1,\,hex}$ and $E_{2,\,hex}$ and their subfractions after NP fractionation (E_1F_1, E_1F_2, E_2F_1, and E_2F_2) were chemically analyzed to determine the recoveries of the individual compounds. This first recovery screening revealed that the spiked compounds eluted mainly in the first SPE fraction ($E_{1,\,hex}$), while no spiked compounds or only very small amounts were detected in the second fraction ($E_{2,\,hex}$). Therefore, the E_2 fractions were not further analyzed. After NP-HPLC fractionation, OH-PBDEs were found in subfraction E_1F_1, whereas OH-PCBs were detected mainly in the E_1F_2 subfraction, where also the endogenous THs were expected.[17] Because relevant analytes were found in both the E_1F_1 and E_1F_2 subfractions and the TT_4 concentration in the plasma extract was too low to interfere with the radioligand T_4*-TTR bioassay (see below), further chemical analyses and bioassay measurements of other samples were restricted to fraction $E_{1,\,hex}$ without NP-HPLC fractionation.

After SPE-LLE, the test compounds in fraction $E_{1,\,hex}$ showed average (±SD) recoveries of 107(±8.1)% for the OHPs, 93.8(±15.5)% for the OH-PCBs, and 93.8(±19.5)% for the OH-PBDEs (Table 2).

For parent PCBs and PBDEs low recoveries were found (~30%, $E_{1,\,hex}$, Table 2). Thomsen et al.[3] reported recoveries of 64−108% for the same PCBs and PBDEs in spiked serum extracted by Oasis HLB through reversed-phase hydrophobic interaction. In our cartridge selection experiment the same type of HLB cartridge was tested, but this material did not provide recoveries above 30% for these compounds (Table S2, Supporting Information). Since the hydroxylated metabolites of PCBs and PBDEs have remarkably stronger TTR-binding affinity than their parent compounds,[4,12,18] no further effort was made to improve recovery of the parent compounds.

The recoveries of the PFASs in E_1 (for which no LLE was done prior to chemical analysis) decreased with carbon atom chain length from 115% to 2%, with acceptable recoveries 85.2(±24.6)% for the most potent, short chain (<8 C-atoms) PFASs, independent of the type of their polar group (Table 2).

Biologically Determined Recoveries. In the TTR-assay, all extracts of plasma spiked with the different compound classes showed activity in accordance with the chemically determined

Table 2. Chemical Recoveries (%±SD) of the Spiking Compounds Determined in the Fraction $E_{1,\ hex}$ of the Spiked Cow Plasma after SPE (n = 3) by GC-MS, GC-μECD, and LC-MS/MS

GC-MS, GC-μECD,	% ± SD	LC-MS/MS	% ± SD	chain length	polar group
2,4,5-TCP	113 ± 9.8	**PFBA**	60 ± 14.9	**4**	**acid**
2,4,6-TBP	110 ± 5.4	**PFPeA**	95 ± 20.1	**5**	
PCP	95 ± 3.8	**PFHxA**	111 ± 1.6	**6**	
triclosan	110 ± 9.2	**PFHpA**	104 ± 7.0	**7**	
Average	**107 ± 8.1**	**PFOA**	83 ± 11.8	**8**	
4-OH−CB 118	97 ± 2.1	**PFNA**	51 ± 9.1	**9**	
4-OH−CB 107	105 ± 11.3	**PFDcA**	24 ± 3.8	**10**	
4′−OH-CB 130	102 ± 8.7	**PFUnA**	12 ± 1.0	**11**	
4-OH−CB 187	71 ± 1.5	**PFDoA**	6 ± 0.2	**12**	
Average	**93.8 ± 15.5**	**PFTrA**	3 ± 0.2	**13**	
BDE 28	32 ± 7	**PFTdA**	2 ± 0.2	**14**	
PCB 153	29 ± 7	**Average**	**50 ± 42.9**		
PCB 138	30 ± 7	PFBS	115 ± 15.1	**4**	**sulfonate**
BDE 49	30 ± 7	PFHxS	87 ± 11.8	**6**	
BDE 47	30 ± 7	PFOS	33 ± 4.4	**8**	
PCB 180	29 ± 7	THPFOS	87 ± 9.6	**8**	
PCB 170	30 ± 7	**Average**	**81 ± 34.3**		
BDE 100	26 ± 6	**PFOSA**	77 ± 10.0	**8**	**sulfonamide**
Average	**30 ± 1.7**	**Average short chain PFASs (<8)**			**85 ± 24.6**
4′−OH-BDE49	120 ± 19.5				
5-OH-BDE47	102 ± 19.3				
4-OH-BDE47	98 ± 18.8				
3-OH-BDE47	76 ± 9.8				
2′−OH-BDE66	73 ± 7.1				
Average	**93.8 ± 19.5**				

Table 3. Overview of the TTR-Binding Activities in Extracts of Spiked Plasma (Fraction $E_{1,\ hex}$; Oasis MCX) Measured in the Radioligand T_4*-TTR Binding Assay[d]

	measured activity in extracts of spiked plasma (n = 3) (μM T_4-Eq in plasma) average ± SD	measured activity in the spiking mixtures (n = 1)[b] (μM T_4-Eq) average	calculated activity[c] (μM T_4-Eq)	recovery relative to measured activity in the spiking mixture (%)	recovery relative to calculated activity (%)
OH-PCBs	0.52 ± 0.02	0.58	0.50	90	104
OH-PBDEs	0.41 ± 0.03	0.49	0.48	84	85
PCBs and PBDEs	n.d.	n.d.	0.003[a]	-	-
PFASs	n.d.	n.d.	0.013[a]	-	-
OHPs	1.51 ± 0.05	1.97	1.61	77	82
combined mixture	2.05 ± 0.08	2.62	2.59	78	79

[a] These values are below the Limit of Detection (LOD), which is 0.016 μM T_4-Eq (~20% T_4* binding to TTR). [b] The spiking mixture was measured in the assay after transfer into DMSO. [c] Calculated activities are determined based on the T_4 REP-factors of the spiking compounds and their actual concentration in the spiked plasma. [d] Results are expressed in μM T_4-Eq in plasma and compared to the measured activity in the spiking mixtures and to the calculated activities as recoveries (%). n.d., not detected; "-", not calculated.

recoveries and with the calculated and measured activities of the spiking mixtures (Tables 2 and 3).

For extracts of plasma samples spiked with OH-PCBs, TTR-binding activities were mainly found in the $E_{1,\ hex}$ fraction, especially in its second subfraction E_1F_2 (Figure 2 C). An activity of 0.52 μM T_4-Eq was found in the $E_{1,\ hex}$, which is in good agreement with the calculated activity (0.50 μM T_4-Eq; 104% recovery) and with the measured activity in the spiking mixtures (0.58 μM T_4-Eq; 90% recovery) (Table 3).

For extracts of plasma samples spiked with OH-PBDEs, TTR-binding activities were also found in the fraction $E_{1,\ hex}$, but in its first HPLC fraction E_1F_1 (Figure 2D). The measured activity in the E_1 (0.41 μM T_4-Eq) was in line with the calculated (0.48 μM T_4-Eq) and measured (0.49 μM T_4-Eq) activities, resulting in recoveries >84% (Table 3).

The activities found in the extracts of plasma samples spiked with the OHPs (1.51 μM T_4-Eq) were in accordance with the measured activity in the spiking mixture (77% recovery) and with the calculated activity (82%; Table 3, Figure 2E). The extracts of

plasma spiked with the combined mixture of OH-PCBs, OH-PBDEs, and OHPs showed an activity of 2.05 μM T_4-Eq, in good agreement with the measured activity in the spiking mixture and with the calculated activity (>78% recovery; Table 3, Figure 2E).

PCBs, PBDEs, and PFASs are less potent TH-disruptors than OH-PCBs, OH-PBDEs, and OHPs and consequently also have lower REP values (Table 1). The calculated total potencies for the extracts of the plasmas spiked with both PCBs and PBDEs (3 nM T_4-Eq) and with PFASs (10 nM T_4-Eq) were below 16 nM T_4-Eq, which is the limit of detection (LOD) in the assay. Consequently, no activity was detected in the extracts of plasma spiked with PCBs, PBDEs, and PFASs (Table 3).

All (sub)fractions of the blanks were tested for competitive TTR-binding activity. Neither the procedure blanks nor the plasma blanks showed any activity. In the extract of the water spiked with OH-PBDEs, a similar activity (0.40 μM T_4-Eq) was found as in extract of the plasma spiked with the OH-PBDEs (0.41 μM T_4-Eq), indicating that the eventually remaining sample matrix constituents do not suppress or enhance the TTR-binding activities of the spiked compounds in the plasma extracts.

Endogenous TT_4 Levels. The presence of endogenous hormones in biota extracts may interfere with the measurement of endocrine disrupting compounds in *in vitro* bioassays.[17,19] Therefore, NP-HPLC fractionation was initially used to separate endogenous hormones from POPs in biota extracts as was previously done in studies on estrogens in blood matrices[20] and androgens and THs in fish tissues.[17] The NP-HPLC elution profile of the spiking compounds (data not shown) revealed that OH-PCBs eluted in the same subfraction (E_1F_2) as the endogenous THs.[17] However, after SPE about 95% of the initial TT4 concentration (65 nM) was removed from the cow plasma. Taking all the concentration and dilution steps in the T4*-TTR binding assay protocol into account, the remaining 3 nM TT_4, determined in the E_1 fraction, corresponds to 5.6 nM T_4-Eq in the assay. According to the T_4 calibration curve, covering a concentration range of 0−1024 nM T_4 (Figure 2A), this concentration was below 16 nM T_4-Eq, which is the LOD in the assay and too low to influence the measured activities in extracts of the spiked samples. In the polar bear plasma, even lower initial TT_4 concentrations (8 and 20 nM, respectively) were measured, again indicating no influence on the bioassay result. Therefore, we did not apply further NP-HPLC fractionation for TH removal from the extracts of other spiked cow plasma samples or nonspiked polar bear plasma samples.

Assuming that ±95% of plasma TT_4 is removed during the SPE procedure, TT_4 levels for e.g., monkey, seal, fish, and dolphin were taken from the literature,[21−24] and their possible influence on the assay was calculated when extracting a 3 mL sample (the sample volume for which the developed method was validated). For all studied species, the calculated T_4-Eqs were already below the lowest T_4 level (16 nM) that caused an effect in the 3-fold diluted extracts (Table 5). So, further dilution will lead to an even lower T_4 level in the extracts with no influence on the bioassay results. This indicates that the contribution of endogenous T_4 does not lead to overestimation of the measured TTR-binding activity in plasma extracts from different species, which emphasizes the broad scope of the SPE-LLE method and its suitability for inclusion in EDA strategies.

Polar Bear Plasma. The SPE-LLE method successfully extracted the biologically active compounds present in the polar bear plasma samples and the activities, known to be partly caused by a suite of chemically determined OH-PCBs, could be measured in the T_4*-TTR binding assay. This is in accordance with a previous study that showed that in polar bear plasma, 4-OH-CB107 had a high binding affinity to TTR.[25] In the first polar bear plasma extract, the calculated TTR-binding activity caused by the OH-PCBs (1.1 μM T_4-Eq) could explain 85% of the actually measured TTR-binding activity (1.3 μM T_4-Eq). In the

Table 4. Measured and Calculated T_4-Eq of the Two Polar Bear Plasma Extracts[a]

		polar bear 1		polar bear 2	
compound	molar T_4 REP-factors	concentration (nM)	T_4-Eq (nM)	concentration (nM)	T_4-Eq (nM)
4-OH-CB107	3.5	10.3	36.1	5.1	17.9
4-OH-CB146	3.5	70.4	246.4	73.5	257.3
3′-OH-CB138	3.3	2.0	6.6	1.0	3.3
4′-OH-CB130	3.7	0.5	1.9	0.3	1.1
3-OH-CB180	1.9	1.9	3.6	1.1	2.1
4′-OH-CB172	3.8	33.2	126.2	42.1	160.0
4-OH-CB187	4.0	117.6	470.4	123.0	492.9
calculated activity			**1.1 μM**		**0.9 μM**
measured activity			**1.3 μM**		**1.5 μM**
% T_4-Eq explained by OH-PCBs			**85%**		**60%**

[a] The calculated activity is determined based on measured OH-PCBs levels and T_4 REP-factors.

Table 5. Total T_4 Levels (nM) Measured in Plasma of Different Species and Their Corresponding Highest T_4-Concentration in the Radioligand T_4*-TTR Binding Assay, under Similar Extraction and Test Conditions As Used in the Present Study[a]

	TT_4 (nM) in plasma	reference	T_4 (nM) in T_4*-TTR binding assay (3-times diluted extract)
cow (n = 1)	65	this study	1.9
polar bear (n = 2)	8−20	this study	0.25−0.6
human (not indicated)	66−181	Azezli et al., 2007[21]	2.1−5.6
seal (n = 1)	2.8−16.3	own unpublished data	0.03−0.17
sea bream (n = 1)	43	Morgado et al., 2007[22]	1.3
dolphin (n = 72)	109−311	Aubin et al., 1996[23]	3.4−9.7
Rhesus monkey (n = 5)	80−112	Sawhney et al., 1978[24]	2.5−3.5
lowest level of thyroxine (T_4) in the assay causing effect:			**16 nM**

[a] A 3 mL plasma sample was reduced to a 40 μL extract in DMSO; diluted 40-times in the assay; 5% recovery of T_4 was expected. These calculated concentrations can be compared to the lowest level of T_4 causing effect in the assay (Figure 2A).

second extract, the calculated activity (0.9 μM T_4-Eq) could explain 60% of the measured activity (1.5 μM T_4-Eq). The measured activities in the polar bear plasma extracts ($E_{1,\ hex}$) were higher than the expected activities based on calculations using the OH-PCB REP values and the measured ΣOH-PCB concentrations (Table 4). This might indicate the presence of other TH-disrupting compounds.

Method Applicability. The good (71−120%) recoveries determined by chemical analysis for various potent TH-disruptors (OH-PCBs, OH-PBDEs, OHPs, and short-chain PFASs except PFBA) in combination with good recoveries (77−104%) determined in extracts of plasma spiked with these compounds in the bioassay demonstrate the applicability of the developed plasma sample preparation method in e.g., an EDA study.

The relative standard deviations (RSDs (%)) obtained for the chemically analyzed recoveries of the different classes of compounds ranged from 1.4 to 24.8%, while for bioassay measurements RSDs of 3.3 to 8.3% were obtained.

After the chemical and biological validation of the SPE-LLE plasma sample preparation method and a pilot study on two polar bear plasma samples, a larger number of polar bear plasma samples (n = 30) have been extracted in collaboration with the Norwegian University of Science and Technology (NTNU), in the framework of the BearHealth-project. Three polar bear plasma samples showing a combination of high competitive TTR-binding activity in the TTR assay with low levels of target analyzed TH-disrupting compounds were selected for further studies to identify the compounds causing the unexplained TTR-binding activities in the samples. This work is currently carried out in a study design based on EDA, using high resolution mass spectrometry to identify the unknown TH-disrupting compounds.

■ ASSOCIATED CONTENT

Supporting Information. Additional information on cow and polar bear plasma samples, on chemical analysis, and on SPE cartridge selection. This material is available free of charge via the Internet at http://pubs.acs.org.

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